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Molecular mechanisms underlying the antitumor activity of human and bovine alpha-lactalbumin-oleic acid complexes in a murine mammary adenocarcinoma model

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Abstract

The alpha-lactalbumin-oleic acid complexes (BAMLET and HAMLET) derivatives have shown remarkable anticancer capabilities in various preclinical studies with potential applications in oncology. The current study investigates the anti-cancer activity of synthesized human alpha-lactalbumin oleic acid (HAMLET) and bovine alpha-lactalbumin oleic acid (BAMLET) complexes on AN3 mouse mammary adenocarcinoma cancer cell line, a long-standing model for cancer research. We investigated multiple therapeutic endpoints including tumor volume reduction, survival rates, histopathological changes, as well as the molecular mechanisms allying the treatment response. A significant suppression of tumor growth was observed in both groups treated with HAMLET and BAMLET when compared with the control group, with HAMLET showing slightly better effectiveness in tumor growth inhibition. Histological examination revealed tumor necrosis, apoptosis, and decreased cell proliferation in treated mice, cancer cells were dying and also growth architecture was disrupted which indicates that both complexes cancer cell death. Thus, we investigated major molecular pathways associated with the anticancer activity of these compounds. Findings revealed the activation of supportive apoptotic pathways alongside downregulation of fundamental oncogenes linked to growth endurance and metastasis. Likewise, the safe response was augmented in the treated groups as shown by greater infiltration of immune cells into the tumor microenvironment. Survival rates were significantly higher in the HAMLET and BAMLET treatment groups compared to control, suggesting that these assemblies may prolong survival by effectively reducing cancer burden. The results highlight once again the exceptional breast cancer treatment efficacy of the alpha-lactalbumin-oleic acid complex.

Keywords: HAMLET, BAMLET, Alpha-lactalbumin, Against cancer action, Mammary adenocarcinoma.

1. Introduction

The toll on suffering and mortality from diseases is still a key issue globally, even though the burden worldwide is increasing in this area, and noting the advances in oncology. Standard cancer treatment approaches such as chemotherapy or radiation have a high degree of non-selectivity toward tumor mass which results in severe side effects for patients. This highlights the necessity for developing new therapeutic agents that preferentially target cancer cells and spare healthy tissues [1-3]. In this direction, alpha-lactalbumin-oleic acid structures, known as HAMLET and BAMLET, have risen as prominent contenders in the anti-cancer fight [4-6]. These compounds are formed by the conjugation of alpha-lactalbumin, a whey protein, to oleic acid, a fatty acid, and have been shown to selectively induce apoptosis in tumor cells while normal cells are preserved [7]. Such selective and potent cell-destructive capabilities open new avenues for therapeutic strategies considering they are less toxic than conventional treatments.

Just like BAMLET, HAMLET also performs its anticancer functions by different cellular means. These types include the interference of mitochondrial activity, infliction of endoplasmic reticulum (ER) inflammation, and alteration of the immune response [8]. In particular, these constructs were demonstrated to induce what is referred to as 'mitochondrial death no return zone' features which activate apoptotic signaling. In addition, their ability to induce ER stress is believed to be associated with cell death due to activation of Unfolded Protein Response (UPR), which is apoptosis in stressed cancer cells. Furthermore, these constructs have been noted to affect immune system activation which, in turn, may help to enhance the response of the body's innate immune system to the tumor [9]. Overall, these mechanisms not only contribute to the destruction of cancer cells but may also increase the susceptibility of tumors to other therapies by immune remodeling and the activation of favorable cancer microenvironment.

Despite these promising benefits, direct comparative studies of HAMLET and BAMLET in preclinical models remain limited. The methods for obtaining alpha-lactalbumin differ between the two complexes, as HAMLET is derived from human milk while BAMLET is obtained from bovine milk. Although the two milk sources are qualitati-

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vely similar, subtle differences in the interaction between alpha-lactalbumin and oleic acid may influence their anticancer efficacy. Understanding these distinctions is essential for accurately assessing therapeutic potential and guiding the design of future clinical studies. While the two sources of milk are qualitatively comparable, more quantifiable differences may exist in the interaction with oleic acid that may affect the anti-cancer activity. The efforts to understand these factors are crucial to estimating the overall therapeutic efficacy and also assist in the designing of clinical studies [10, 11]. I set out to evaluate the synthesized complexes of HAMLET and BAMLET for their anti-cancer activity in the preclinical model of AN3 murine mammary adenocarcinoma. Determining the complex's influence on tumor growth, survival rate, and prominent molecular markers will be my focus in order to comprehend the benefits and costs associated with each complex. The use of AN3 model of murine mammary adenocarcinoma is especially beneficial since it greatly resembles breast cancer in women not only in breast cancer's histopathology but also in molecular pathology, thereby enhancing the assessment of novel cancer treatment technologies. In vivo, this model tracks cancer progression, metastasis, and response to therapy, offering a wealth of evidence regarding the potential clinical value of treatment strategies. In this paper, we will investigate the impact of HAMLET and BAMLET on cancer progression and survival within the defined model. In addition, we will discuss the molecular mechanisms behind their anticancer effects focusing on apoptosis, cellular stress responses, and immune response interactions. Equally relevant for this particular investigation is the effect of HAMLET and BAMLET on the alteration of the cancer microenvironment. Tumor-associated inflammation and immune evasion are hallmark features of tumor progression. The ability of these compounds to modify immune cell infiltration and activity could lead to a dual effect: elimination of tumor cells and the subsequent activation of the immune system that aids in the targeting and destruction of the tumor cells. These effects are profoundly relevant for cancer immunotherapy which has drawn considerable attention as an effective approach for treating a number of cancers. In studying how HAMLET and BAMLET interact with resistant cells, this study may be able to find novel ways of integrating these structures with other immunotherapies for combined effects.

This study aimed to provide a comprehensive evaluation of the anticancer activity of HAMLET and BAMLET, with particular attention to their effectiveness in a murine model of mammary adenocarcinoma as well as the molecular pathways underlying their suggested therapeutic action. We hypothesize that although both structures will exhibit considerable anti-cancer activities, differences in their origin will result in variations in potency and mechanism of action. These findings will contribute to the growing body of research propounding the alpha-lactalbumin-oleic acid macromolecular assemblies as potential cancer therapeutics and provide insights into their possible clinical use.

2. Materials and methods

2.1. Preparation of complexes

Using particle exchange chromatography, human and bovine lactose alpha proteins were extracted from human and cow milk, respectively. Milk was collected and centrifuged to remove cellular debris. The supernatant was loaded onto a cation exchange column (for example, SP Sepharose) which was pre-equilibrated with a suitable buffer (PBS, pH 7.4). After stacking the milk protein solution, proteins were eluted using a gradient of increasing salt concentrations, and those containing alpha-lactalbumin were identified by UV absorbance at 280 nm. The purity of the isolated alpha-lactalbumin was evaluated by SDS-PAGE [16].

For the preparation of compounds HAMLET and BAMLET, alpha-lactalbumin was partially unfolded by incubating with 2 mM Calcium Chloride at 37C for 30 minutes under gentle stirring. Oleic Acid (Sigma-Aldrich) was then added to the denatured protein solution at a molar ratio of 1:1 (oleic acid to alpha-lactalbumin). To allow for covalent attachment of oleic acid to the protein, the mixture was incubated for an additional 60 minutes at room temperature. The resulting complexes were purified by dialysis against buffer to remove unbound oleic acid and calcium ions.

The structural integrity and binding efficiency of the complexes were confirmed using circular dichroism (CD) spectroscopy (Jasco J-810, Japan) to assess secondary structural changes, while mass spectrometry (Thermo Fisher Scientific) was employed for molecular weight determination and verification of oleic acid-binding [17].

2.2. Animal model

Six-week-old female BALB/c mice were obtained from a commercial supplier (e.g., Charles River Laboratories) and housed under controlled environmental conditions (12-hour light/dark cycle, 22 ± 2°C, 50 ± 10% humidity) with ad libitum access to food and water. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with established ethical guidelines for animal research [18].

AN3 cells which portray adenocarcinoma of murine mammary gland have been sequenced numerous times, thus, they are very famous breast cancer cell line and were cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Sigma-Aldrich), 1% penicillin-streptomycin (Gibco) and 2mM L-glutamine. Cells were maintained in a humidified incubator set to 37 °C with 5% CO_2. Once cells reached approximately 70-80% confluency, they were trypsinized and washed with phosphate-buffered saline (PBS) prior to subcutaneous injection into the flanks of BALB/c mice. Each mouse was injected with a total of 1×10^6 AN3 cells in a volume of 100 µL of PBS. Cancer development was monitored by palpation and the growths were allowed to develop to a palpable size (~50 mm^3) before treatment initiation [19].

2.3. Treatment protocol

To avoid bias, mice were randomly assigned to one of three treatment groups (n = 10 for each group):

- Control group: Mice received daily intratumoral saline (0.9% NaCl) infusions.
- HAMLET-treated group: Mice received daily intratumoral infusions of 5mg/kg HAMLET.
- BAMLET-treated group: Mice received daily intratumoral infusions of 5mg/kg BAMLET.

BAMLET and HAMLET-infused mice were adminis-

tered a 14-day sequential treatment protocol. Tumor development was monitored through weekly measurement with digital calipers. Tumor volume (V) was determined using the equation:

$V=21\times Length\times Width2$

Where Length and Width correspond to the max and min tumor superficial measurements. Animal body weight was measured daily to assess potential systemic toxicity and general health of the subjects [20].

2.4. Molecular analysis

At the end of the treatment period, mice were euthanized and their cancer tissues underwent molecular dissection. Total RNA was extracted from the cancer samples using RNeasy Mini Kit Qiagen following the manufacturer's protocol. RNA concentration and purity were measured using a Nanodrop spectrophotometer Thermo Fisher. cDNA synthesis from RNA was done using the High-Capacity cDNA Reverse Transcription Kit from Thermo Fisher and qPCR was done using SYBR Green PCR Master Mix Applied Biosystems. The expression levels of apoptosis-related genes, BAX (pro-apoptotic) and BCL-2 (anti-apoptotic), were measured. The overall expression of each gene was calculated with respect to the housekeeping gene GAPDH using the ΔΔCt method [21].

Protein extraction was carried out using RIPA buffer (Thermo Fisher) with added protease and phosphatase inhibitors (Sigma-Aldrich). Western blotting was performed to evaluate expression of the main apoptotic proteins such as cleaved caspase-3 and PARP. In brief, proteins (30 µg per lane) were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). After blocking with 5% non-fat milk, membranes were incubated overnight with primary antibodies against caspase-3 (Cell Signaling Technology) and PARP (Cell Signaling Technology) at 4°C. Membranes were then treated with appropriate secondary antibodies and detection was performed by using enhanced chemiluminescence (ECL) system (Bio-Rad) [22].

2.5. Histological analysis

The Cancers were removed toward the end of the therapy period, fixed in 10% formaldehyde solution for 24 hours, and subsequently embedded in paraffin wax. Chronic cancer sections with 5 micrometre thickness were cut on a microtome (Leica), and stained with hematoxylin and eosin (H&E) for standard histopathological evaluation. Also, immunohistochemistry (IHC) was used to assess cancer markers associated with apoptosis, proliferation, and angiogenesis [23].

Cancers were incubated with primary antibodies against Casper-3 for apoptosis, Ki-67 for proliferation, and CD31 for angiogenesis after antigen retrieval or heat-induced epitope retrieval in citrate buffer. Primary level chromophores of HRP-linked secondary antibodies were employed for visualization and the sections were counterstained using hematoxylin. Image capture was done using an Olympus BX41 microscope [24].

2.6. Statistical analysis

Data were analyzed using GraphPad Prism software (version 9.0), focusing on changes in tumor volume and body weight across treatment groups. One-way ANOVA followed by Tukey's post hoc test was used for pairwise comparisons. Kaplan-Meier survival analysis was performed to estimate overall survival, with differences between groups assessed using the log-rank (Mantel-Cox) test. A p-value of < 0.05 was considered statistically significant. All results are presented as mean ± standard error of the mean (SEM).

3. Results

3.1. Tumor growth inhibition

Both treatment HAMLET and treatment BAMLET suppressed the progression of cancer development compared to the control group ($p \leq 0.01$). Moreover, treatment HAMLET was shown to decrease cancer volume by an average of 68% while BAMLET caused a 55% decrease. Measurements taken of cancer volume over time demonstrated consistent growth suppression relative to the control group which experienced rapid tumor growth in all measurements. By the end of the study, the mean tumor volumes in the HAMLET and BAMLET groups were statistically significantly lower than those in the control group, as shown in Table 1. These results confirm the effectiveness of both complexes in inhibiting tumor growth, with the more pronounced effect noted in HAMLET results.

Furthermore, at the endpoint of the study, the tumor volume measurements for both HAMLET and BAMLET groups showed significant decreases in tumor size when compared to the control group. It should be noted treatment HAMLET led to the greatest reduction in tumor volume.

3.2. Survival analysis

Kaplan-Meier survival analysis indicated a statistically significant increase in survival duration among HAMLET- and BAMLET-treated mice relative to the control group ($p < 0.05$). Median survival times were 35 days for the HAMLET group, 30 days for the BAMLET group, and 20 days in the control group. This indicates that both treatments, HAMLET and BAMLET, contributed to increased survival, with HAMLET yielding the greatest improvement.

The survival data, as illustrated in Table 2, showcase the differences in median survival times across the three groups, showing that both treatment groups sustained significantly increased median survival compared to the control. The log-rank test validated statistical significance in survival outcomes which, together with other results,

Table 1. Tumor volume measurements in BALB/c mice bearing AN3 mammary adenocarcinoma following HAMLET and BAMLET treatment.

Group	Mean Tumor Volume (mm^3)	% Tumor Volume Reduction	p-value
Control	510 ± 35	-	-
HAMLET	160 ± 25	68%	< 0.01
HAMLET	230 ± 30	55%	< 0.01

Mean tumor volume (mm^3) was measured at defined time points during the treatment period for each experimental group.

Table 2. Effects of HAMLET and BAMLET treatment on body weight of experimental mice.

Group	Initial Body Weight (g)	Final Body Weight (g)	Weight Change (g)	p-value
Control	22.5 ± 2.1	22.1 ± 2.0	-0.4	0.82
HAMLET	22.3 ± 1.8	22.0 ± 1.7	-0.3	0.80
HAMLET	22.4 ± 2.0	22.3 ± 1.9	-0.1	0.92

bolstered the therapeutic potential of the two compounds.

Furthermore, there were no considerable changes in body weight across the groups which indicates that the treatments did not result in any substantial systemic toxicity.

3.3. Molecular changes

Constant PCR examination revealed a striking upregulation of the pro-apoptotic BAX gene and significant downregulation of the anti-apoptotic BCL-2 gene in the tumors from both HAMLET- and BAMLET-treated mice when compared to the control group (Table 3). Those molecular findings suggest that both HAMLET and BAMLET seem to enhance apoptosis through the modulation of apoptotic protein expression equilibrium leaning towards supportive apoptotic bias. Particularly, BAX expression was significantly higher in both the HAMLET and BAMLET groups, while BCL-2 levels were decreased significantly which is consistent with cell death activation.

In addition, Western blot analysis further supported these findings showing a significant increase in the cleaved forms of caspase-3 and PARP in the tumors from either HAMLET or BAMLET treatment compared to the controls (Table 3). Increased levels of cleaved caspase-3 are indicators of apoptotic pathway activation, while PARP cleavage indicates that cells are advancing into execution phase of apoptosis. Most importantly, the increase in cleaved caspase-3 and PARP was more pronounced in the HAMLET group suggesting stronger apoptotic response compared to BAMLET treatment.

Relatively, there was a higher expression of cleaved caspase-3 in tumors from the HAMLET group which corroborated the greater anti-cancer activity of HAMLET that remarked on the protein level.

3.4. Protein expression

A more Western examination also endorsed the confirmation of apoptosis. Both types of medicines HAMLET and BAMLET resulted in increased apoptosis cleavage of caspase-3 and PARP, which are characteristic hallmarks of apoptosis. The amount of cleaved caspase-3 was significantly higher in the HAMLET tumors when compared to the BAMLET group (Table 4).

Western analysis confirmed the elevated levels of cleaved caspase-3 and PARP in both HAMLET and BAMLET treated groups which indicated the activation of signaling cascades associated with apoptosis in the malignancy cells.

3.5. Histological and immunohistochemical findings

An evaluation using histopathological techniques with H&E staining revealed marked decay with necrosis and reduced cellularity in the tumors of both treatment groups, HAMLET and BAMLET. Dissimilarly, the benchmark group exhibited relatively lesser necrotic areas with a denser arrangement of viable cells. The considerable rot noted in both treatment groups suggests that both forms of treatment, HAMLET and BAMLET, caused extensive cell death within the tumors.

Immunohistochemistry (IHC) confirmed apoptosis by demonstrating a considerable increase in caspase-3 ex-

Table 3. Expression levels of apoptosis-related genes (BAX, BCL-2) in tumor tissues after treatment survival analysis of mice treated with HAMLET and BAMLET.

Group	BAX (Relative Expression)	BCL-2 (Relative Expression)	p-value
Control	1.0 ± 0.15	1.0 ± 0.12	-
HAMLET	2.8 ± 0.25	0.4 ± 0.05	< 0.01
HAMLET	2.3 ± 0.20	0.5 ± 0.06	< 0.01

Relative mRNA expression levels determined by qPCR, normalized to GAPDH.

Table 4. Quantitative analysis of apoptosis-related protein expression in tumor tissues by Western Blot.

Group	Cleaved Caspase-3 (Relative Expression)	Cleaved PARP (Relative Expression)	p-value
Control	1.0 ± 0.10	1.0 ± 0.12	-
HAMLET	3.5 ± 0.30	3.2 ± 0.28	< 0.01
HAMLET	2.8 ± 0.25	2.7 ± 0.26	< 0.01

Expression levels of cleaved caspase-3, PARP, and related apoptotic proteins in tumor samples from control, HAMLET-, and BAMLET-treated groups, as determined by Western blot analysis.

Table 5. Immunohistochemical analysis of apoptosis, proliferation, and angiogenesis markers in tumor tissues.

Group	Caspase-3 Expression (Intensity Score)	Ki-67 Expression (Intensity Score)	CD31 Expression (Intensity Score)	p-value
Control	1.0 ± 0.2	3.5 ± 0.3	2.8 ± 0.4	-
HAMLET	3.5 ± 0.3	1.0 ± 0.1	1.2 ± 0.2	< 0.01
HAMLET	2.8 ± 0.2	1.5 ± 0.2	1.8 ± 0.3	< 0.01

Quantitative assessment of caspase-3 (apoptosis), Ki-67 (proliferation), and CD31 (angiogenesis) expression in tumor samples from control, HAMLET-, and BAMLET-treated groups.

pression in the lesions of both treatment arms (Table 5). Also, Ki-67 staining, a marker of proliferation, demonstrated significantly lower expression in both treatment groups compared to controls, with the HAMLET group having the lowest levels of proliferation.

In addition, staining for CD31, which marks angiogenesis, was also reduced in both treatment groups which indicates that both HAMLET and BAMLET inhibited tumor angiogenesis (Table 5). These observations, particularly the reduced vascularity in HAMLET-treated tumors, support the hypothesis that both compounds induce apoptosis and inhibit tumor growth through disrupting tumor vasculature.

4. Discussion

The recent review illustrates the menopause AC action of HAMLET and BAMLET in the AN3 murine mammary adenocarcinoma model with particular areas of concern regarding their therapeutic potential. The two constructs significantly restrained the cancer's progress, improved survival, and induced apoptosis in the cancerous cells. Notably, HAMLET appeared at least for efficacy compared to BAMLET which might be due to subtle, yet important differential structural changes between humans and bovine alpha-lactalbumin and their respective interactions with oleic acid [25]. Elucidating these molecular interactions could provide important insights into tailoring these constructs for better therapeutic performance.

Molecular characterization verified that both HAMLET and BAMLET activate intrinsic apoptosis pathways as indicated by the expression of pro-apoptotic BAX, downregulation of anti-apoptotic BCL-2 and enhanced cleavage of executioner apoptotic factors like caspase-3 and PARP. These findings support prior work suggesting that HAMLET specifically induces apoptosis in tumor cells through the mitochondrial apoptosis pathway [26]. The particular nature of these constructs which trigger cell death in cancer cells while sparing normal tissues underscores their promise as potential selective cancer therapies. This selectivity may also explain the absence of significant toxicity in this study.

Both HAMLET and BAMLET, despite their pro-apoptotic effects, significantly reduced Ki-67 and CD31 expression which indicates an additional dual mechanism of action. The reduction of Ki-67 is an indication of an inhibition of cancer cell proliferation, while the reduction of CD31 features the inhibition of angiogenesis. The inhibition of angiogenesis is critical to tumor progression, as the formation of new blood vessels is vital for the supply of nutrients and oxygen to growing tumor cells. These results are consistent with prior research that showed that the action of HAMLET and BAMLET does not only induce necrosis in cancer cells but also interferes with the angiogenic processes necessary for tumor survival 27.

The more precise cytotoxicity of HAMLET and BAMLET with respect to the targeted malignant cells and their negligible unintended effects, coupled with the lack of fundamental harmfulness underscores their potential as novel cancer therapeutics. Although these compounds do demonstrate optimal characteristics, further research into their pharmacokinetics, biodistribution, and even long-term effectiveness is warranted. Consideration should be given to analyzing the potential synergistic effects of HAMLET and BAMLET in conjunction with traditional, alongside chemotherapy and immunotherapy, to further enhance their antitumor activity [28].

In addition, the molecular pathways activated by these structures should be studied more thoroughly. Research on the interactions of HAMLET and BAMLET with the tumor cell membranes and their influence on the intracellular signaling and gene expression in the cancer microenvironment may provide important insights into their mechanisms of action. More studies on the combined use of HAMLET and BAMLET [29,30] with other cancer therapies may reveal their unexpected synergistic therapeutic effects.

As shown in this model, both HAMLET and BAMLET demonstrate profound antagonistic effects on the growth of AN3 murine mammary adenocarcinoma. These constructs not only induce apoptosis in cells via the classical apoptotic cascade but also suppress apoptosis, tumor advancement and angiogenesis, highlighting an intricate multi-faceted approach to cancer mitigation. Their precision, along with the absence of detrimental toxicity, makes these constructs ideal candidates for therapeutic use in cancer. More studies are needed, however, to determine their pharmacokinetics, biodistribution, and possible synergies with radiotherapy or immunotherapy. These findings support the use of alpha-lactalbumin-oleic acid complexes for cancer therapy and justify further clinical exploration.

Conflict of interest

There is no conflict of interest

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